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Research article

Anti-aging effects of Korean Red Ginseng (KRG) in differentiated embryo chondrocyte (DEC) knockout mice

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ABSTRACT

Background: The circadian rhythm is the internal clock that controls sleep-wake cycles, metabolism, cognition, and several processes in the body, and its disruption has been associated with aging. The differentiated embryo chondrocyte (*Dec*) gene is related to circadian rhythm. To our knowledge, there are no reports of the relationship between dec gene expression and KRG effect. Therefore, we treated *Dec* gene knockout (KO) aging mice with KRG to study anti-aging related effects and possible mechanisms.
Methods: We evaluated KRG and expression of *Dec* genes in an ototoxicity model. *Dec* genes expression in livers of aging mice was further analyzed. Then, we assessed the effects of DEC KO on hearing function in mice by ABR. Finally, we performed DNA microarray to identify KRG-related gene expression changes in mouse liver and assessed the results using KEGG analysis.
Results: KRG decreased the expression of *Dec* genes in ototoxicity model, which may contribute to its anti-aging efficacy. Moreover, KRG suppressed *Dec* genes expression in liver of wild type indicating inhibition of senescence. ABR test indicated that KRG improved auditory function in aging mouse, demonstrating KRG efficacy on aging related diseases.
Conclusion: Finally, in KEGG analysis of 238 genes that were activated and 158 that were inhibited by KRG in DEC KO mice, activated genes were involved in proliferation signaling, mineral absorption, and PPAR signaling whereas the inhibited genes were involved in arachidonic acid metabolism and peroxisomes. Our data indicate that inhibition of senescence-related *Dec* genes may explain the anti-aging efficacy of KRG.

1. Introduction

In 2016, global life expectancy averaged 72 years and has been growing rapidly over the past few years [1]. In 2019, there were 703 million people worldwide aged 65 years or older. By 2050, the number of older people is expected to double to 1.5 billion [2]. Globally, life expectancy has increased the importance and interest in anti-aging research for healthy aging [3].

Aging implies the loss of organ and tissue function. There are various theories on the biological causes of aging, such as molecular damage, cellular defects, and oxidative stress [4,5,24]. Age-related hearing loss known as presbycusis is due to degenerative changes in the cochlear nerve cells. Recently hearing loss has become a common sensory impairment and is no longer a personal problem but a social health problem [6].

The aging mechanism is related with oxidative stress, which can induce age-related diseases. Reactive oxygen species (ROS) can cause mRNA damage and lipid/protein oxidation and consequently result in a reduction in mitochondrial function, which eventually generates more oxidative stress. The decline in mitochondrial function and oxidative stress reaction in aging may contribute to age-related disease [24].

Circadian rhythm is the endogenous order that organizes behavior and physiological systems under the 24-hour dark/light

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cycle and is controlled by the suprachiasmatic nucleus (SCN) located in the hypothalamus [7]. The SCN is the principal circadian clock in mammals contemporized by the external environment to the photic neural input pathway called the retinohypothalamic tract [8]. Alterations in the neurochemical and electrophysiological output of the SCN are one of the causes of aging in mammals and results in temperature, locomotor, and drinking behavior changes in rodents [9]. Moreover, the transplantation of fetal SCN into an aging host is associated with interesting results. Neuroendocrine substrate from aged animals persisted in responding to diurnal cues to maintained proper signals, demonstrating that the SCN plays an important role in aging and longevity [10]. The molecular machinery of the circadian rhythm correlates with clock and period genes (PER); the autofeedback loop generated by the CLOCK/BMAL heterodimer acts as a positive regulator and the internal clock PER/CRY are the negative regulators. In addition, *Dec1* and *Dec2*, encoding the transcription factors basic helix–loop–helix (bHLH), increased the promoter activity in the same way as PERs. Furthermore, *Dec1* expression is motivated by transforming growth factor-β, nerve growth factor, cAMP, and hypoxia which are causes of senescence, therefore *Dec* genes might be the key in modeling the molecular mechanisms of aging [11]. Red ginseng is reported to improve age-related hearing, vestibular dysfunction, and learning and memory in aged animal models [12,13]. In this study, we have identified the effects of Korean Red Ginseng (KRG) and its role on expression levels of the *Dec1* and *Dec2* genes in neomycin (NM)-induced toxicity in HEI-OC1 cells. Besides, we have assessed the expression level of *Dec1* and *Dec2* genes in KRG treated aging mice. Aging wild type (WT) mice and aging DEC KO mice were used to confirm hearing function and cognitive ability after treatment with KRG. Finally, microarray analysis was used to confirm KRG-related gene expression changes and associated with antiaging potential mechanisms of KRG.

2. Materials and methods

2.1. Cell viability (MTT assay)

The House Ear Institute-Organ of Corti 1 (HEI-OC1) mouse auditory cell line was cultured in permissive conditions (33°C with 10% CO_2) with high-glucose Dulbecco's modified Eagle medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) containing 50 U/mL INF-γ (Peprotech, Seoul, Korea) without antibiotics and 10% FBS (fetal bovine serum; Welgene, Gyeongsangbuk-do, Korea) as described previously [14].

The cells were subcultured in 96-well flat-bottom plates at a density of 1×10^4 cells/well. c After treatment at 33°C, cells were incubated with MTT (Duchefa Biochemie, Amsterdam, the Netherlands) solution (0.5 mg/mL), added to the cell culture media for 4 hours. After incubation, removed the solution and added 100 μL of dimethyl sulfoxide to each well to dissolve the formazan crystals. Absorbance was evaluated at 570 and 630 nm (Synergy HT, BioTek Instruments, Winooski, VT, USA), and the average OD in the control cells was taken as 100% viability.

2.2. Quantitative PCR (qPCR)

The cells were subcultured in 6-well culture plates at a density of 1×10^6 cells/well. The cells were incubated for 24 hours and then pretreated for 1 hour with KRG (100 μg/mL), after that cotreatment of KRG at the same concentration with 15 mM NM for 24 hours. After treatment at 33°C, total RNA was extracted from cells with Trizol reagent (Thermo Fisher Scientific, Seoul, Korea) according to the manufacturer's protocol. The relative mRNA expression level was measured by qPCR and β-actin was used to normalize mRNA expression. Total RNA (1 μg) was reverse-transcribed with Reverse Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. The qPCR was performed in 10 μL reactions containing 5 μL of SYBR Select Master Mix (Applied Biosystems, Thermo Fisher Scientific), 1 μL of cDNA template, 1 μL of forward primer (10 pmol), 1 μL of reverse primer (10 pmol), and 2 μL of RNase free water. The qPCR parameters are: initial denaturation at 95°C for 5 min, followed by 45 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 20 s, and then 73°C for 5 min. The expression of genes was analyzed by the $2^{-\Delta\Delta Ct}$ method. Primer sequences are listed in Table 1.

2.3. Animals

Dec 2−/− (DEC KO) mice were generated by the Ingenious Targeting Laboratory (Stony Brook, NY, USA). Animals were housed under 12 hours light-dark cycle, given *ad libitum* access to food and water and maintained at temperature (23.0 ± 1.0°C) and humidity (50.0 ± 5.0%).

2.4. Ethical statement

All experimental procedures using mice were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication, No. 80-23, revised 1996) and the Animal Care and Use Committee of Kyung Hee University (KHUASP(SE)-15-17), Republic of Korea.

2.5. Auditory function evaluation

Auditory function were evaluated using channel recording (GSI Audera, Viasys Healthcare, Conshohocken, USA). Animals were anesthetized using xylazine (Bayer, Germany), ketamine (Yuhan Corporation, Korea), and saline solution (JW Pharmaceutical, Korea) (1.1:4:4.9, respectively) injected intramuscularly before the test. Mice were located in an electrically and acoustically sheltered sound emaciation booth (TCA-500D, Sontek, Korea). The stimuli were transported through earphones (Etymotic ER-EA). Electrode needles were located subcutaneously at the vertex of the skull, the postauricular region and in the lower back.

Experimental mice were divided into 4 groups (n = 4/group) and treated orally once daily with 0.3 mL of distilled water (control WT and control DEC KO) or 200 mg/kg of KRG (KRG 200 WT and KRG 200 DEC KO).

2.6. Evaluation of auditory brainstem response (ABR)

Hearing thresholds were measured using ABR at 30 days after KRG treatment. For the ABR recordings, alternating 8 and 16 kHz tone bursts (TBs) (rise-plateau-fall; 2-1-2 cycles) were delivered through earphones (Etymotic ER-3A) at a rate of 20.1 stimuli/s and high-frequency transducers. Physiological filters were set to pass electrical activity between 100 and 3,000 Hz. Each mouse has a monaural response that was recorded and averaged in a 10.24 ms time window. 1,000 sweeps were collected. For threshold determination of the ABR test, the 8 and 16 kHz TBs were decreased in 5 dB steps near the hearing threshold. The assessment of ABR

Table 1
Primers for qPCR

Gene	Forward Sequence	Reverse Sequence
β-Actin	GAA GAG CTA TGA GCT GCC TGA	TGA TCC ACA TCT GCT GGA AGG
Dec1	CCA AAG UGA UGG ACU UCA A	UUG AAG UCC AUC ACU UUG G
Dec2	CAG GCC TCC TAC CTT GTC AC	TGT CCC AAA TGA TTC ACC AA

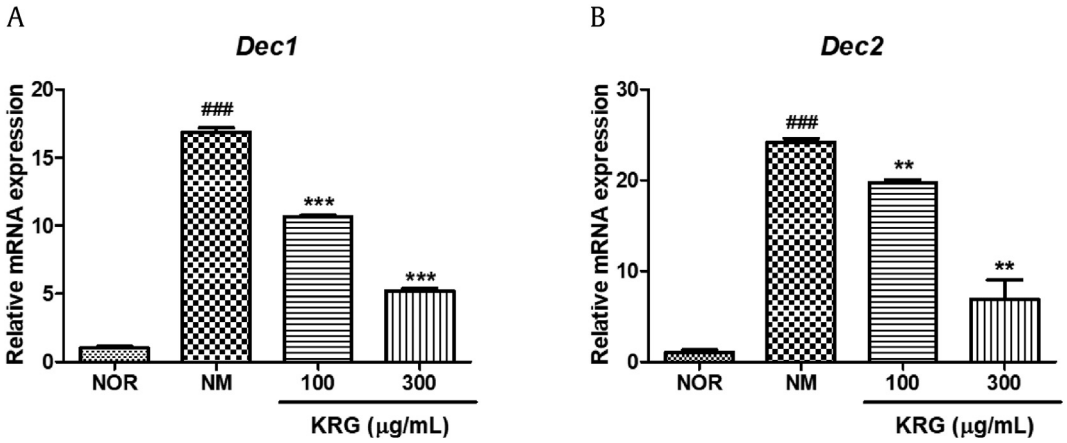

Fig. 1. KRG decreases DEC mRNA expression in HEI-OC1 cells. *Dec1* A and *Dec2* B were significantly decreased by KRG compare with NM. Data are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (significant differences between the NM and KRG treated groups). $^{\#\#\#}P < 0.001$ (significant differences between the NOR and NM group). NOR: normal group, NM: neomycin induced group, 100: KRG100 μg/mL treated group, 300: KRG300 μg/mL treated group.

parameters was based on hearing thresholds and interpeak latencies of waves I–V at a peak sound pressure level (pSPL) of 80 dB.

2.7. Evaluation of auditory middle latency response (AMLR)

Central auditory function was determined using AMLR at 30 days after KRG treatment. To measure of the AMLR, rarefaction clicks (0.1-ms duration) were transported via earphones at a rate of 9.1 stimuli/s. Filters were set to pass activity between 10 and 250 Hz. The average of 250 sweeps was determined in a 70-ms time window. The AMLR parameters were assessed with absolute Pa wave latencies at 80 dB pSPL. and amplitudes were marked from the negative peak to the following positive peak (Na–Pa).

2.8. Behavioral test

The Y-maze equipment consisted of three arms made of plastic form a "Y" shape. The walls of the arms were 15.5 cm high, the Y-maze scheme was based on published protocols [15]. Briefly, mouse was placed into one of the maze arms and allowed to explore the maze with one of the arms closed for 15 min. Mouse was placed to the start arm. Then, the mice were allowed to explore freely all arms of the maze for 8 min. The number of entries into each arm and the first choice of entry were recorded.

2.9. Microarray

Trizol reagent was used to extract total RNA from mouse liver. The cDNA library was prepared and amplified using WTA2 (Sigma-Aldrich). The cDNA was purified by Purelink (Invitrogen). The cDNA was fragmented with DNase I and biotinylated with end-transferase using a GeneChip Human Mapping 10K 2.0 Assay Kit (Affymetrix). Each sample target is hybridized to a mouse genomic 430 PM array at 45°C for 16 hours. Hybridized samples were washed and stained with GeneAtlas Fluidics Station (Affymetrix). Arrays were scanned at the Gene Atlas Imaging Station (Affymetrix). The Gene Ontology (GO) database and statistical tests utilizing DAVID were used to analyze the genes according to *P* value. The GO analysis was performed on the genes with fold change 1.5 and an adjusted *P* value of 0.05 or less. KEGG was analyzed using EnrichR [16].

2.10. Statistical analysis

The data were expressed as mean ± standard error of the mean (SEM) using GraphPad Prism (version 5). Statistical comparisons was determined using repeated one-way analysis of variance (ANOVA) with post-hoc tests. Statistical significance considered was $P < 0.05$.

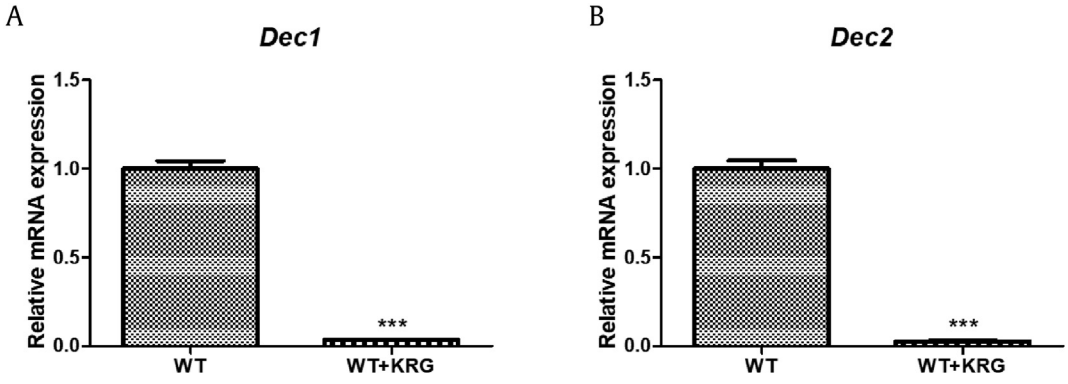

Fig. 2. KRG inhibits the Dec gene in the aging mice liver. *Dec1* A and *Dec2* B were significantly decreased by KRG compare with the control (WT). Data are presented as the mean ± SEM. $^{***}P < 0.001$.

3. Results

3.1. Dec1 and Dec2 expression in neomycin-induced ototoxicity in HEI-OC1 cells

To confirm *Dec* expression on ototoxicity in HEI-OC1 cells, we performed qPCR and targeted the *Dec1* and *Dec2* genes. *Dec1* and *Dec2* significantly increased in NM treated HEI-OC1 cells. The KRG treated group significantly suppressed the changes in the expression of *Dec1* and *Dec2* that were up-regulated by NM, indicating that KRG has efficacy against oxidative stress (Fig. 1). These results suggest that the anti-aging mechanism of KRG might be related with the reduced expression of *Dec1* and *Dec2*.

3.2. Dec1 and Dec2 expression in aging mice

To confirm *Dec* expression in aging mice, we performed qPCR and targeted *Dec1* and *Dec2* in the liver. In 24-month-old WT mice treated with 200 mg/kg KRG, *Dec1* and *Dec2* were significantly decreased (Fig. 2). KRG reduced the expression level of *Dec1* and *Dec2* suggesting an anti-aging mechanism.

3.3. Efficacy of KRG on auditory function in Dec KO mice

Hearing thresholds of 25-month-old WT mice were around 64 dB in 8 kHz and 16 kHz. However, 25-month-old DEC KO mice significantly reduced the threshold in 8 kHz and 16 kHz ($p < 0.01$) compared to the control. Furthermore, the hearing thresholds of 200 mg/kg KRG treated the 25-month-old DEC KO mice significantly decreased in 8 kHz and 16 kHz ($p < 0.01$, $p < 0.001$) compared to the 200 mg/kg KRG treated WT mice (Fig. 3A and B).

The effect of KRG on central auditory function was also assessed through the AMLR test. After 30 days of treatment, KRG treated DEC KO mice exhibited a significant increase ($P < 0.05$) in the amplitude between Na and Pa peaks compared to KRG treated WT mice (Fig. 3C and D). Thus, indicating that KRG treatment improved central auditory function. The results showed that DEC KO prevents hearing damage and KRG has a beneficial effect on increasing the improvement of hearing function.

3.4. Y-maze

The Y-maze assessment is suitable for memory and route-learning abilities, where we investigated the exploratory behavior of the mice [17,18]. Testing occurs in a Y-shaped maze with three same arms at equal distance from each other. After the mouse are introduced to the center of the maze, they have the freedom to access all three arms. If the mouse chooses a different arm from the one it arrived, this choice is called an alteration. This is considered the correct response, whereas returning to the previous arm is considered an error. The total number of arm admissions and the sequence of admissions are recorded. KRG treated DEC KO mice visited significantly more arms than KRG treated WT mice (Fig. 4).

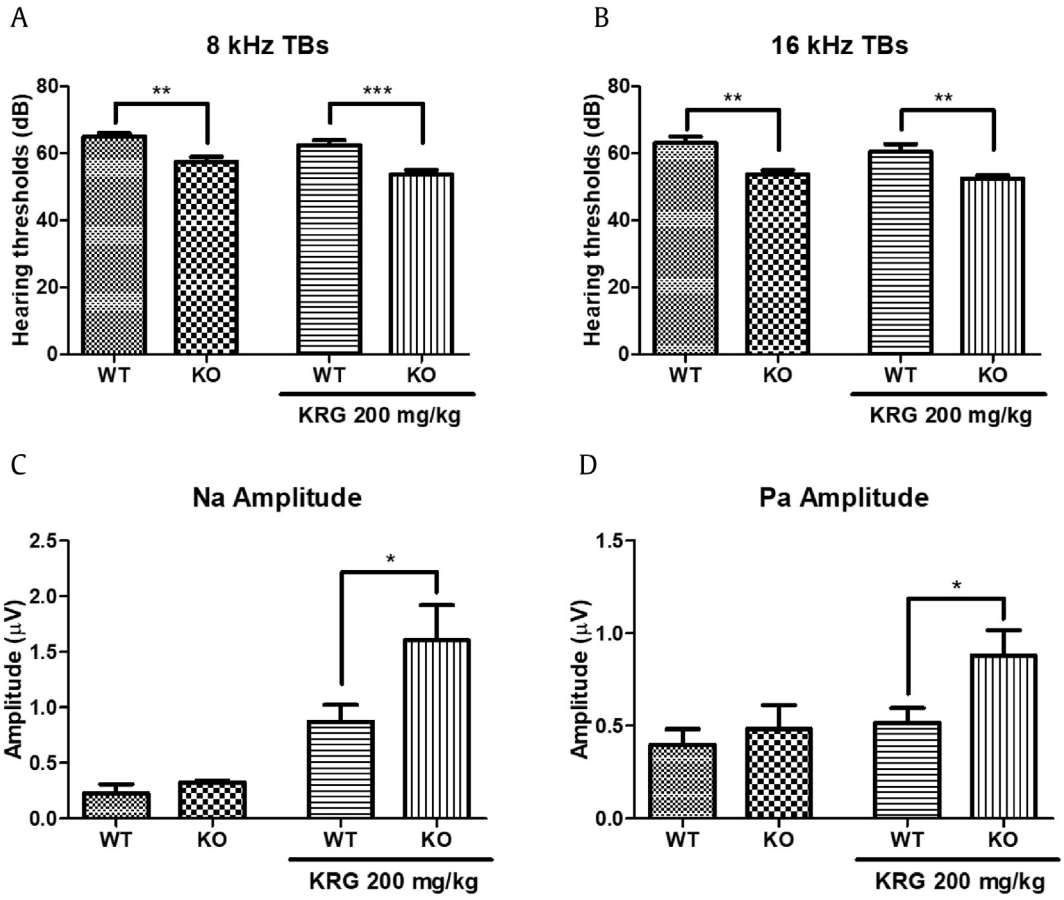

Fig. 3. KRG efficacy in age-related hearing loss. Auditory brainstem response (ABR) Hearing threshold with A, 8 kHz and B, 16 kHz tone bursts in 25-month WT and DEC KO mice. Auditory middle latency response (AMLR) C, Na amplitude and D, Pa amplitude in 24-month WT and DEC KO mice. Data are presented as the mean ± SEM. $^{*}P < 0.05$. $^{**}P < 0.01$, $^{***}P < 0.001$.

3.5. Differential gene expression induced by KRG

KRG activated 238 genes and inhibited 158 genes. GO was analyzed to identify the biological processes using differentially expressed genes.

To identify the functional pathways, KEGG analysis was performed using differentially expressed genes. The genes that were up-regulated by KRG treatment were enriched in the proliferation signaling pathway, mineral absorption, amino sugar metabolism, PPAR signaling pathway, and retinol metabolism. The genes that were down-regulated by KRG treatment were enriched in metabolic pathways, arachidonic acid metabolism, peroxisome, and the AGE-RAGE signaling pathway (Fig. 5).

4. Discussion

In this study, we focused on the anti-aging effect of KRG and its possible mechanisms. The DEC1 gene or BHLHE40, Bhlhb2, Stra13 and Sharp-2, is one of the clock genes, which is involved in circadian rhythm regulation, cell proliferation and differentiation,

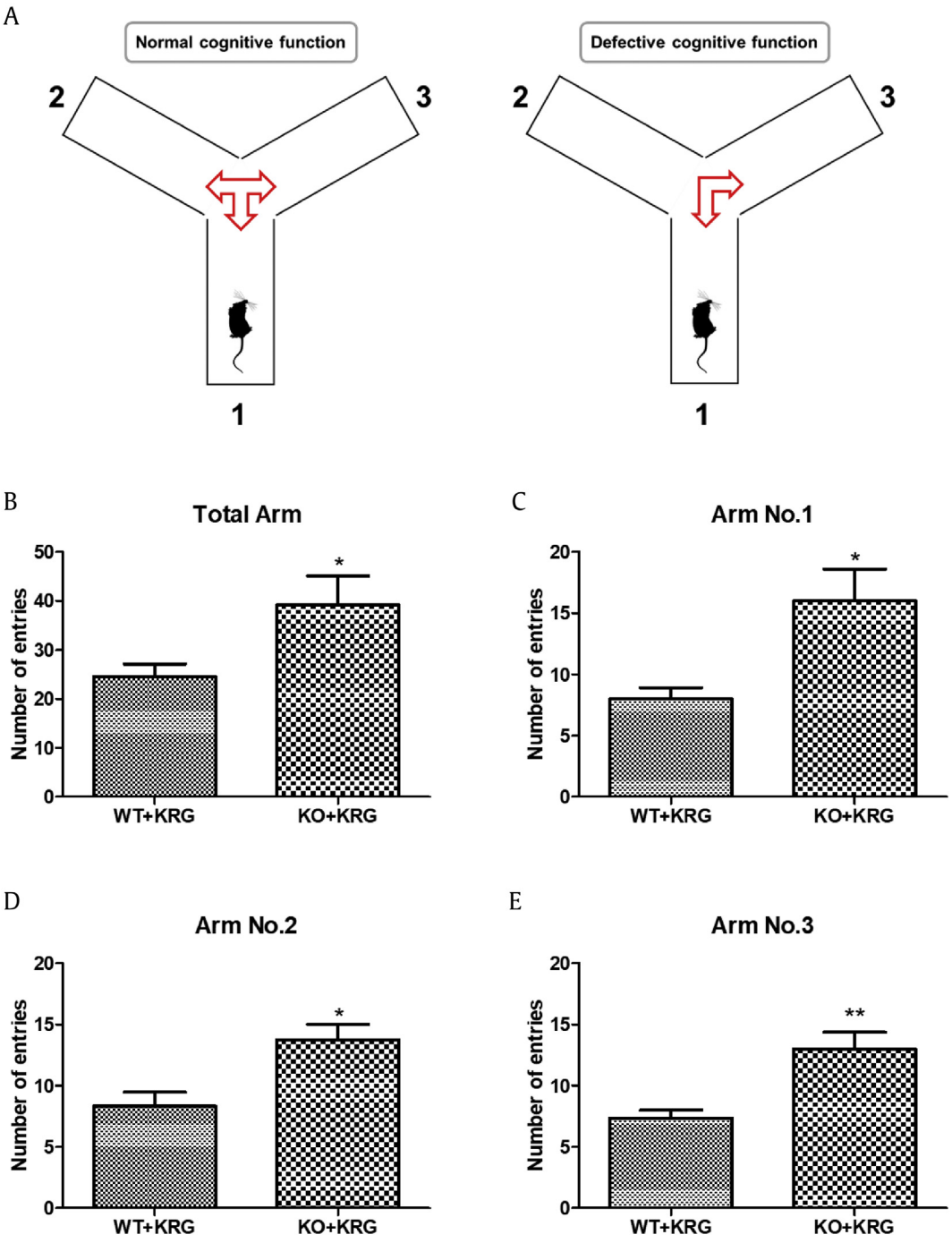

Fig. 4. Response to Y-maze (three arms) for KRG treated WT mice and KRG treated DEC KO mice. A, Schematic representation of Y-maze. B, Number of entries into each arm. C, Number of entries into arm No. 1. D, Number of entries into arm No. 2. E, Number of entries into arm No. 3. Data are presented as the mean ± SEM.*$P < 0.05$, **$P < 0.01$.

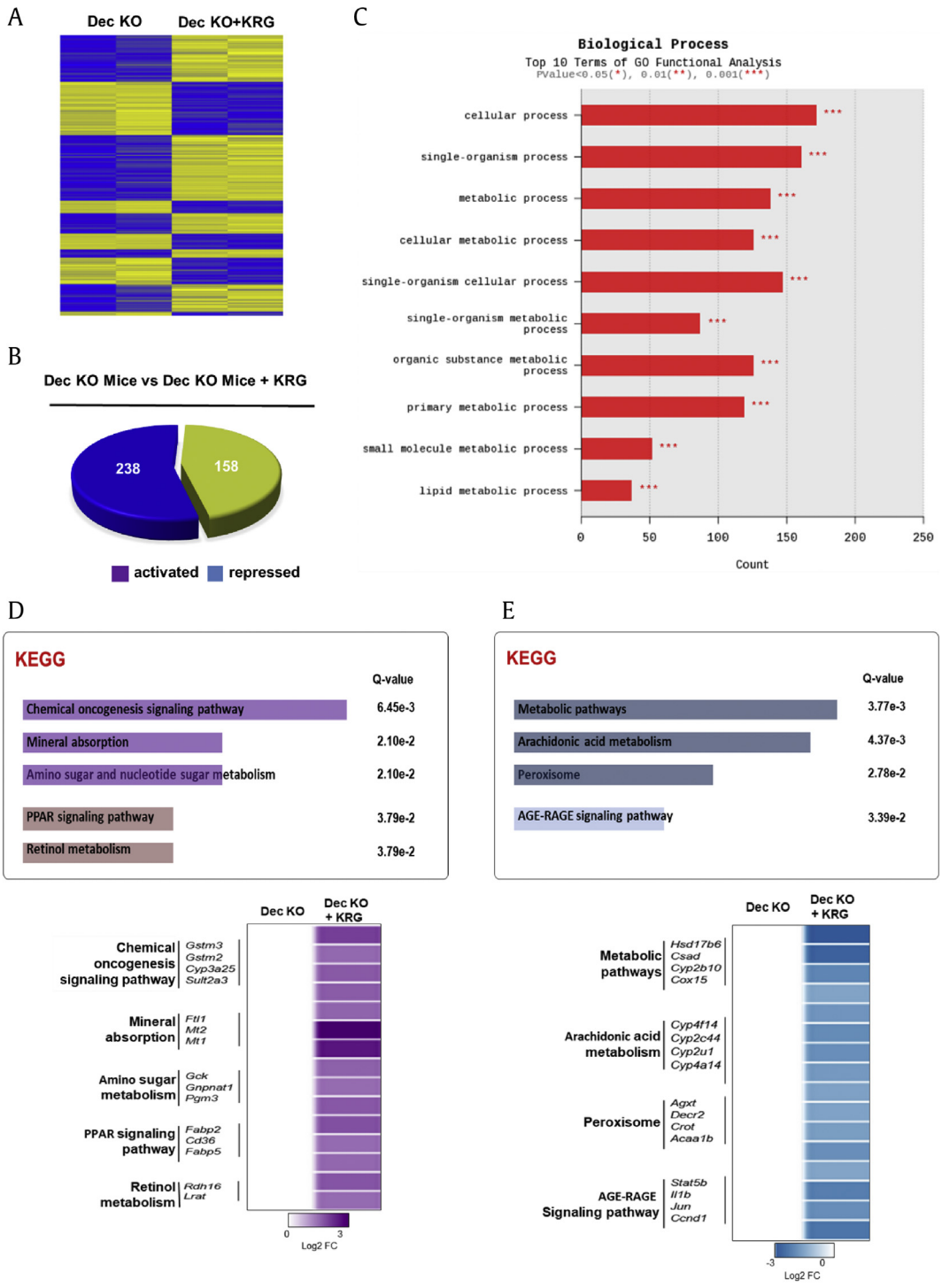

Fig. 5. Microarray analysis of differential gene expression profiles from the livers of 24-month-old DEC KO mice treated with KRG. A, Heat map based on microarray analysis of gene expression in DEC KO mice. B, Graph showing the results of microarray analysis for the KRG-regulated gene set. C, Analysis of the genes that differed in GO analysis revealed a term that was involved in liver development, which had a statistical significance of $P < 0.01$. Gene function by Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. D, Up-regulated pathways of genes influenced by KRG and E, down-regulated pathways of genes influenced by KRG.

apoptosis and cellular senescence [19]. The DEC2 gene or BHLHE41, BHLHB3 and SHARP1, is believed to be involved in the control of circadian rhythm and cell differentiation [20]. Thus, DEC1 and DEC2 have crucial roles in the regulation of circadian rhythms by suppression of CLOCK/BMAL transactivation [20]. Moreover, disturbances in the circadian rhythm have been reported to cause behavioral and physiological dysfunctions, diabetes, metabolic syndrome and aging [19]. A large number of physiological events depend on the circadian rhythm, and it has been hypothesized that its function declines with age, which explains much of the physiological deterioration associated with aging. Thus, the process of aging and circadian rhythm are closely associated [21–23].

Oxidative stress is long-established among the theories that explain the mechanism of aging, which is also involved in the generation and progression of several diseases including age-related hearing loss [24]. Currently, there are no reports of the relationship between DEC gene expression and age-related hearing loss. Since DEC genes is well established as a senecence marker, we thus hypothesized that its expression might be important for the hearing loss progression. Furthermore, KRG has shown sufficient evidence of its potential anti-aging effect [25,26], however the mechanisms involved in such effect have not been fully elucidated. Therefore, with this study we want to demonstrate that KRG improvement on auditory function in aging mice might be explained through DEC gene inhibition.

We examined the expression of *Dec1* and *Dec2* according to the efficacy of KRG on NM-induced ototoxicity in HEI-OC1 cells. Cisplatin and aminoglycoside antibiotics can produce ROS, which can cause hearing impairment [27]. A previous study reported that cisplatin treatment increases *Dec*1 gene expression [28]. As we expected, our data showed that NM significantly increased *Dec1* and *Dec2* expression in HEI-OC1 whereas KRG decreased their expression, which confirms that the expression of the *DEC1/2* genes may be important in the antiaging efficacy of KRG.

To further confirm the anti-aging action of KRG, we examined *Dec1* and *Dec2* expression in the aging liver of 25-month-old WT mice treated with KRG. The expression of *Dec1* and *Dec2* significantly decreased in KRG treated mice. These results showed once again that KRG decreases *Dec1* and *Dec2* expression, which might lead to an anti-aging effect.

Age-related hearing loss (presbycusis) gradually happens as we grow older, and is one of the most common conditions affecting older adult. We investigated KRG efficacy on hearing function in WT and DEC KO aging mice.We treated both WT and DEC KO mice with KRG and confirmed the influence of KRG. In particular, KRG treatment on DEC KO mice increased the Na and Pa amplitude in an AMLR test. This result suggests that KRG improved hearing function in age-related hearing loss. The efficacy of KRG in different forms of sensorineural hearing loss including age-related hearing loss is well known [29], our results confirm previous reports. Additionally, since the relationship between the expression of the DEC gene and age-related hearing loss has not been reported to date, in this study we demonstrate as we hypothesized, that its expression is important for the progression of hearing loss. The results have revealed that the auditory function in the DEC KO aging mice was not as impaired as the WT, and that this auditory improvement was further increased when the mice were treated with KRG.

In addition, we performed a Y-maze cognitive function test. Red Ginseng extract and its compounds are known to improve cognitive impairment when evaluated through a behavioral test such as the Y-maze or Morris maze [30–32]. Moreover, DEC1/2 genes ko mice have previously report to improve cognitive processing behavior [33]. In the aging mouse model, our data indicate that KRG has a synergistic effect on cognitive impairment in DEC KO mice.

Finally, we confirmed differential gene expression in the liver of DEC KO mice treated by KRG. The heat map showed that 238 genes were activated and 158 were repressed.

Aging is a result of random molecular damage. It causes an imbalance in oxidative stress, inflammation, and apoptosis, resulting in tissue dysfunction. Thus, oxidative stress plays an important role in the aging of all organs.

We found that the glutathione S-transferase (GST) related genes *Gstm2* and *Gstm3* were up-regulated by KRG. The upregulation of GST might improve aging through an antioxidant effect by protecting cells against lipid peroxidation. Therefore, KRG improved the regulation of aging by enhancing GST [34,35].

Moreover, mineral absorption was also increased by KRG via the upregulation of the *Mt1* and *Mt2* genes. Metallothioneins (MT) protect cells from oxidative stress. Thus, an increase of MTs can reduce ROS through the activation of metal ions, cytokines, and growth factors leading to healthy aging [36–39].

KRG treatment also up-regulated vitamin A or retinol metabolism by increasing the expression of retinol dehydrogenase 16 (*Rdh16*) and lecithin retinol acyltransferase (*Lrat*) genes. Retinol metabolism plays a role key in maintaining clock function and also contributes in the circadian regulation of the cellular redox state. Thus, KRG improves circadian function, which has been associated directly with aging problems.

KRG up-regulated glucokinase (*Gck*), which is associated with the amino sugar metabolism pathway. Amino sugar metabolism and an improvement in glucose metabolism are related to the reduced process of aging. KRG improves glucose metabolism which has been reported as a potential intervention in aging.

In addition, KRG up-regulated the fatty acid-binding protein 2 (*Fabp2*) and the cluster of differentiation 36 (*CD36*) genes, which are related with the PPARγ signaling pathway. The PPARγ pathway is already reported to provide protection from oxidative stress, neuroinflammation, metabolic disturbances, altered neuronal structure, and function, which decreases the aging process [40,41].

Lastly, KRG decreased the expression of the AGE-RAGE signaling pathway-related interleukin 1 beta (*Il1β*) and Jun proto-oncogene (*Jun*) genes, which are both involved in tissue damage, the progression of disease and complications, inflammation, and the apoptosis process, commonly seen in the aging mechanism. Thus, compelling evidence associates KRG with anti-aging mechanisms.

In conclusion, KRG significantly reduced the expression of *Dec* genes, which induced cellular aging on NM-induced HEI-OC1 cells and aging mouse models. The recovery of hearing function and improvement of cognitive ability were confirmed in KRG treated aging DEC KO mice and we confirmed changes in gene expression in these mice using microarray analysis. Our results demonstrate that the mechanism of anti-aging efficacy involving KRG might be explained by several pathways including increased antioxidant defense and PPAR signaling, increased glucose, and retinol metabolism, which helps to maintain circadian rhythm function [42], increased protection against lipid peroxidation and ROS generation, decreased expression of senescence-related proteins, and the AGE-RAGE signaling pathway.

Conflicts of interest

The authors have no conflicts of interest to declare.

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